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In Vitro Biosynthesis of Mouse Hair Keratins Under the Direction of Follicular RNA

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A hair follicle-enriched fraction from neonatal C57BL/6J mouse skin has been obtained by a newly-developed preparative procedure. RNA isolated from this source directs protein synthesis in a cell-free translation system derived from rabbit reticulocytes. Translation products in the molecular weight range of keratins (45,000–70,000 *Mr*) are encoded by RNA species which sediment at 18S on sucrose density gradients. Proteins of 63K, 59K, 58K, 47K and 46K *Mr* were identified as keratins on the basis of electrophoretic mobilities identical with authentic hair keratins and by immunoprecipitation with keratin antiserum.

Hair growth, which occurs only during the anagen portion of the hair cycle, is controlled by regulatory processes which have not been completely defined. It is known that the duration of hair growth depends upon a number of factors such as species, sex, body site, and age [1–3]. Marked responses of the cycle to various hormones have been well-established [4–7] and a variety of experimental approaches have been taken to define the regulatory mechanisms involved [8–16]. We have chosen to investigate the control of hair growth by examining the messenger RNAs which encode the family of filament-forming, constituent proteins of hair, the keratins. These molecules span the molecular weight range of 45,000–70,000 and, together with a less prevalent class of lower molecular weight, nonfilament-forming proteins, form the major structures of the mature hair shaft.

Autoradiographic studies have demonstrated that the bulk of protein synthesis directly related to the production of hair occurs in the deeper portion of the follicle [17]. Accordingly, methods for the isolation of this portion of the tissue have been developed. The technique utilized by most investigators involves epilation to harvest the hair roots. Earlier studies [18–24] used this method to harvest hair roots from which ribosomes and polysomes were subsequently isolated and used to direct cell-free protein biosynthesis. Recently, Ward and Kasmarik [25] have applied such techniques to the isolation of RNA from wool follicles and demonstrated its ability to direct keratin biosynthesis in a cell-free translation system.

Because of the availability of a large number of mutants with qualitative and quantitative abnormalities of hair growth, we have chosen to use mice for our current studies. We have isolated RNA active in protein synthesis from the animals using plucked anagen hairs as the RNA source but have found unsatisfactory variation in the amount of deeper follicular material harvested. We, therefore, have developed a new technique which ensures complete harvest of the lower follicular material and permits us to isolate synthetically active messenger RNA. In this paper, we describe the isolation of this RNA and verify the presence and biological activity of the species which encode hair keratins.

MATERIALS AND METHODS

Animals

Neonatal C57BL/6J mice (9 days old, male and female) were used for all studies. Breeding colonies were established in order to provide a readily available supply of animals [26].

Isolation of RNA

Mice were sacrificed by occipital trauma. Dorsal skin was removed and quick-frozen with liquid nitrogen while spread flat, epidermal side down, on a Dry Ice-cooled metal plate. A #10 scalpel blade was used to scrape the tissue to the level at which pigmentation was lost from the remaining material. The material removed was collected directly into liquid nitrogen.

A modified guanidine hydrochloride extraction [27] technique was used. All operations were performed at 0–4°C unless otherwise stated. Buffers were adjusted at 25°C. Scrapings were homogenized in GSA buffer (10 ml/g scrapings—5 M guanidine hydrochloride, 2% sarkosyl, 100 mM sodium acetate, pH 5) with a tissumizer (Tekmar Co., Cinn., Ohio). Precipitation with ethanol (0.5 vol, overnight, −20°C) followed by centrifugation (27,000 × *g*, 30 min) yielded a pellet which was resuspended in GE buffer (5 ml/g scrapings—6 M guanidine hydrochloride, 25 mM EDTA, pH 7.25) and adjusted to 100 mM sodium acetate, pH 5. The pellet obtained from subsequent ethanol precipitation of the extract was resuspended in TS buffer (3 ml/g scrapings—100 mM Tris·HCl, pH 8, 2% sarkosyl) and twice dialyzed against 100 vol of NEST buffer (100 mM NaCl, 5 mM EDTA, 0.5% sarkosyl, 100 mM Tris·HCl, pH 8) for 1.5 hr. Digestion with Proteinase K (0.1–0.2 mg/g scrapings) for 30 min at room temperature was followed by 2 extractions with an equal volume of phenol:chloroform (1:1). The aqueous phase was adjusted to contain 100 mM sodium acetate, pH 5, and "nucleic acids" were precipitated by addition of ethanol (2 vol, overnight, −20°C). The pellet obtained from centrifugation was resuspended in water (0.7 ml/g scrapings) and reprecipitated with 2 vol of 4.5 M sodium acetate, pH 6. Resuspension in water (0.35 ml/g scrapings) followed by precipitation with 2 vol of ethanol and centrifugation yielded an "RNA" pellet. After removal of residual ethanol under reduced pressure, the RNA was dissolved in water and stored at −20°C. RNA concentrations were determined from absorbance measurements at 260 nanometers, assuming an absorbance of 25 for a 1 mg/ml solution.

RNA was also isolated by this procedure from dorsal hair roots obtained by plucking with forceps.

All glassware used for the procedures was either treated with 0.05% diethylpyrocarbonate (DEP) or washed with 1 M KOH followed by water rinses to eliminate exogenous ribonuclease activity. Buffers were treated with DEP (0.001 volumes) and were kept at 0–4°C overnight before use.

Sucrose Gradient Fractionation of RNA

The aqueous RNA solution, prepared from the hair root-enriched fraction described above, was denatured by heating at 75°C for 3 min followed by rapid cooling on ice. Aliquots containing 125 μg of RNA were layered over 11.2 ml 10–40% linear sucrose gradients in 10 mM NaCl, 1 mM EDTA, and 10 mM Tris·HCl, pH 7.4. Centrifugation was

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Abbreviations:
DEP: diethylpyrocarbonate
PAP: peroxidase-antiperoxidase

at 35,000 rpm at 4°C for 15 hr in a Beckman SW 41 rotor. Gradients were fractioned on an ISCO model 640 density gradient fractionator and RNA was recovered by ethanol precipitation. RNA from each fraction was dissolved in equal volumes (typically 50 microliters) of water and stored at −20°C.

Cell-free Translation of RNA

A messenger RNA-dependent rabbit reticulocyte translation system was prepared as described by Pelham and Jackson [28]. Standard 13 microliter assays containing 1–2 micrograms of RNA and 10 microcuries of ^{35}S-methionine (1,000–1,400 Ci/mmol; Amersham) were incubated for 1.5 hr at 30°C.

Urea-extractable ^{35}S-methionine-labeled Hair Root Proteins

Mice were sacrificed 8 hr after subcutaneous injection of ^{35}S-methionine (250 microcuries) on the dorsum. Dorsal hairs were plucked with forceps, with tension exerted only in the direction the hairs exit from the skin in order to maximize harvest of deeper follicular material.

Serial extractions modified from the procedure of Vermorken, Wetterings, and Bloemendal [29] were used to obtain hair root proteins. The procedure was expanded to include the following sequential fractions: water-soluble, 8 M urea-soluble, 8 M urea/50 mM 2-mercaptoethanol-soluble, and 2% SDS/50 mM 2-mercaptoethanol-soluble. The bulk of the radiolabelled, trichloroacetic acid-precipitable material was found in the urea-soluble fraction (5–10-fold more radioactivity than in the other fractions).

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

A discontinuous slab gel system of the type described by Laemmli [30] was used. Translation product mixtures were routinely treated with RNase A prior to application to discharge labeled transfer RNAs. Molecular weight marker tracks were cut from the gels and stained with Coomassie Blue. The remainder of each gel was prepared for fluorography [31,32] and exposed against either Kodak XR or XAR film at −80°C.

Keratin Antiserum

Rabbit antiserum to keratin was prepared and given to us by M.E. Gilmartin. It was raised against rat epidermal keratin which had been purified by repeated reconstitution of filaments [33].

Indirect Immunofluorescent Staining of Cells and Skin Sections with Keratin Antiserum

Cells from the mouse keratinocyte cell-line Pam 212 developed by Yuspa et al [34] were a gift of R. Crowe and D. Rifkin. They were grown on coverslips, fixed with methanol at −20°C and stained as described by Sun and Green [35]. Frozen sections (8 μ) of skin were fixed with ethanol at −20°C and stained in a similar fashion. Antiserum and preimmune serum were used at a dilution of 1:50.

Reactivity of Urea-extractable Hair Root Proteins with Keratin Antiserum

Urea-extractable hair root proteins were separated into constituent components by gel electrophoresis and then transferred electrophoretically to a nitrocellulose sheet [36]. The nitrocellulose sheet was treated with either keratin antiserum or preimmune serum at 1:50 dilution, following pretreatment with a 3% bovine serum albumin solution and 4% normal goat serum to reduce nonspecific binding. Bound antibodies were coupled to a peroxidase-antiperoxidase (PAP) complex by a bridging antibody (goat anti-rabbit-IgG) [37]. Reaction of the bound PAP with 3,3-diaminobenzidine was used to visualize reactive bands. The distribution of ^{35}S-methionine was visualized by autoradiography with Kodak XAR film.

Immunoprecipitation of Translation Products

Translation mixtures containing products encoded by the 18S RNA from the hair root-enriched skin fraction were reacted with keratin antiserum and preimmune serum as described by Fuchs and Green [38]. Immune complexes were isolated by treatment with formalin-fixed, protein A-containing *S. aureus* (Cowan I strain) as described by Kessler [39]. Precipitated translation products were analyzed by SDS-PAGE and visualized by fluorography as described above.

RESULTS

Tissue Preparation and Isolation of RNA

As an effective extraction procedure for the isolation of intact RNA from skin had been developed previously in our laboratory [40], the major consideration at the outset of this study was how to most effectively obtain a tissue preparation enriched in hair roots. Preliminary experiments using plucked dorsal anagen hairs as the source of RNA gave low yields (approximately 5 μg per mouse). Microscopic examination (data not shown) of the hairs and of histologic sections of the denuded skin revealed that few hairs were harvested intact and that most of the deeper material was left behind in many follicles. The significant limitation in yield of RNA imposed by starting with only a small portion of the potentially available material prompted design of an alternate approach.

A histologic section of normal mouse skin (Fig 1*A*) shows the relevant portion of the hair follicle situated in the deeper portion of the skin, just above the muscle layer and below the dermis, surrounded mostly by adipocytes. The only potential nonfollicular source of RNA encoding keratins is the epidermis. Thus, in the design of a new preparative procedure, it was critical for us to avoid contamination from the epidermis. This was readily accomplished by scraping frozen dorsal skin from the underside (see Methods). This provided a hair root-enriched fraction of the skin, leaving behind the bulk of the dermis and all of the epidermis (Fig 1*B*). Dermal contamination was minimal unless the tissue was scraped past the point at which a loss in pigmentation was first noted.

Yields of RNA were typically 100 μg per animal or about 500 μg/g of scrapings. Yields of RNA from roots obtained from the same amount of dorsal skin as used in the new technique were approximately 5 μg per animal.

Translation Products of Sucrose Gradient-Fractionated RNA

The unfractionated RNA from the hair root-enriched skin fraction encoded a broad range of different size proteins, including prominent species in the keratin molecular weight range (Fig 2, *track T*). In general, a parallel increase in the size of translation products was seen with RNA fractions of sequentially larger sedimentation coefficients (Fig 2, *tracks 5–13*). Most of the RNA which encoded products in the keratin molecular weight range was found in fractions 8 and 9, as was the bulk of the 18S ribosomal RNA. Thus, the RNA species which encoded these proteins also have an average sedimentation coefficient of approximately 18S.

Analysis of Translation Products by SDS-PAGE

Translation products encoded by the 18S RNA (*fractions 8 and 9*, Fig 2) from the hair root-enriched fraction were compared by SDS-PAGE with those encoded by the RNA from plucked hairs and with the urea-extractable hair root proteins (Fig 3). The products encoded by the 2 RNA samples were qualitatively very similar (*tracks 3 and 4*). However, quantitative differences as demonstrated by variable intensities of some bands were observed. This may reflect better harvest of RNA

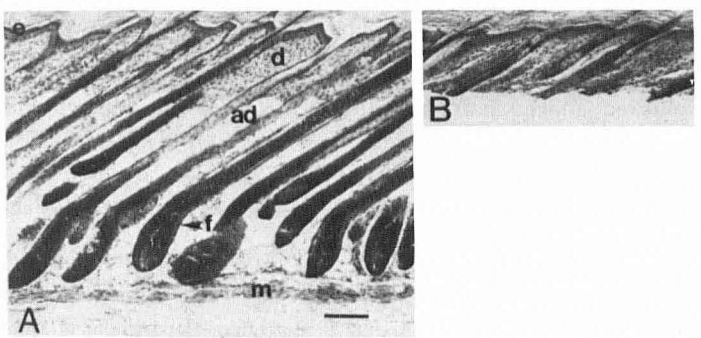

FIG 1. Sections of 9-day-old mouse skin. Skin was fixed with formalin, embedded in paraffin, cut into 6 μ sections, and stained with hematoxylin and eosin. (*A*) Intact skin and (*B*) portion of skin remaining after harvesting hair root-enriched fraction. (*ad*—adipose tissue, *d*—dermis, *e*—epidermis, *f*—hair follicle, and *m*—muscle) *Scale bar* (——) 100 μ.

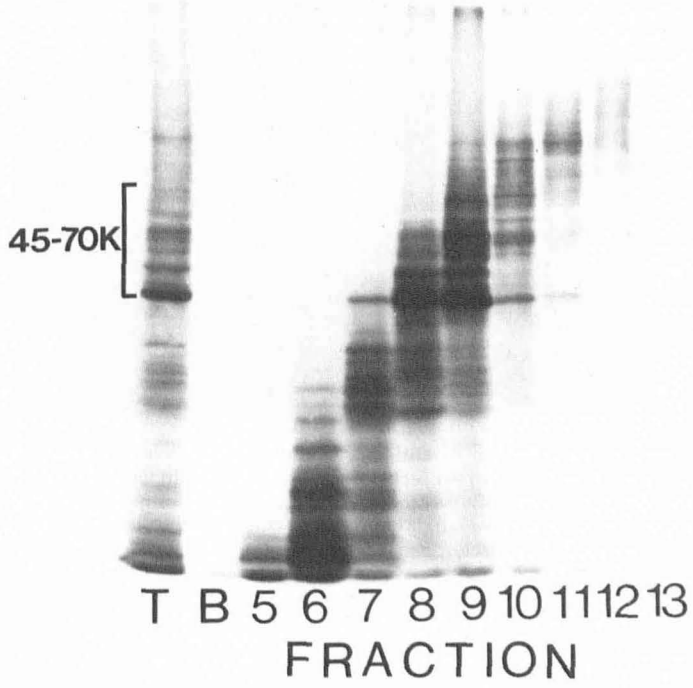

FIG 2. Translation product profile of sucrose gradient-fractionated RNA. Total RNA was fractionated on 10–40% sucrose gradients and recovered by repeated ethanol precipitation. The various fractions were used to direct protein synthesis in a nuclease-treated rabbit reticulocyte lysate system using ^{35}S-methionine to label the products. The products were analyzed by SDS-PAGE and visualized by fluorography. *T*, translation products from "total" unfractionated RNA; *B*, "blank" translation (no added RNA); *5–13*, translation products of RNA fractions with sequentially larger sedimentation coefficients. 18S ribosomal RNA was found in *fractions 8 and 9*. The "keratin molecular weight range" (45,000–70,000 *M*r) is marked by the *bar*.

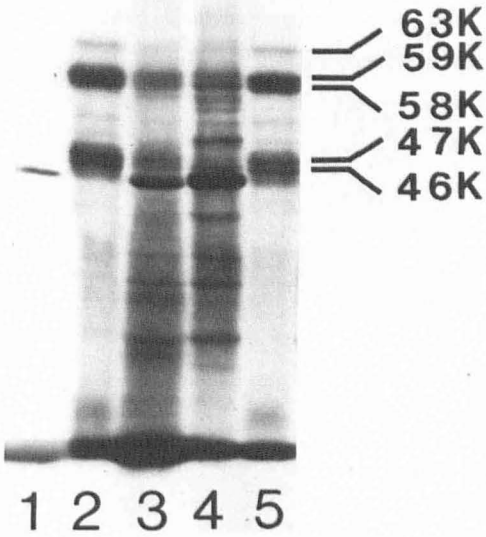

FIG 3. Comparison of translation products with urea-extractable hair root proteins by SDS-PAGE. Bands were visualized by fluorography. *1*, "Blank" translation (no added RNA); *2 and 5*, ^{35}S-methionine-labeled urea-extractable hair root proteins; *3*, translation products of RNA isolated from epilated hairs; *4*, translation products of RNA isolated from hair root-enriched portions of skin and subsequently size-fractionated (*fractions 8 and 9* from Fig 2). Relative molecular weights of major components common to all samples are indicated. (Amount of radioactivity loaded in *tracks 1–5*: 19,000; 28,000; 88,000; 33,000; 28,000 cpm, respectively).

from the deeper portions of the follicle or contamination from nonfollicular tissue. Nevertheless, all major products encoded by RNA prepared from plucked hair roots were found in our preparation and major species in-common with the urea-extractable hair root proteins included those of 63K, 59K, 58K, 47K, and 46K *M*r. This suggests that a similar population of mRNAs was obtained from both sources and a large proportion of the proteins encoded by the 18S RNA was hair proteins.

Specificity of Keratin Antiserum

In order to identify keratins in both the urea-extractable hair root proteins and the translation products, an antiserum to rodent keratin was used. Its specificity for keratin was verified by staining cells from the mouse keratinocyte cell-line Pam 212. The filamentous staining pattern observed (Fig 4) was unchanged after pretreatment of the cells with 5 μg/ml of colcemid (data not shown). It is therefore unlikely that the pattern was due to structures such as microtubules or vimentin filaments [41,42].

It is equally unlikely that the staining was due to microfilaments as can be seen from the data reproduced in Fig 5. *Track 1* is an autoradiogram showing the urea-extractable hair root proteins which were labeled with ^{35}S-methionine. The actin-containing band (*a*) was clearly resolved from the other constituents and it was not reactive with keratin antiserum (*track 2*). We interpret these results to mean that the antiserum was, indeed, reactive with keratin.

Reactivity of the proteins at 63K, 58K, 47K, and 46K *M*r with the antiserum (Fig 5, *track 2*) suggests that they are keratins. The appearance of a reactive protein at 62K (*upper arrow, track 2*) which was not radiolabeled suggests that it was not synthesized during the 8 hr of *in vivo* incubation (see Methods) or that it contained no methionine. The hair protein at 59K

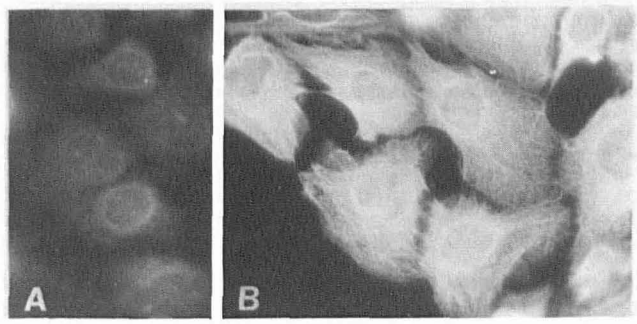

FIG 4. Filaments in mouse keratinocytes of the Pam 212 cell line visualized by indirect immunofluorescent staining with keratin antiserum. *A*, Preimmune serum and *B*, immune serum.

was not reactive with the antiserum in this assay although a less prominent radiolabeled band at 57K (*lower arrow, track 2*) did react. Preimmune serum was not reactive (data not shown).

The follicular distribution of proteins reactive with the keratin antiserum is shown in Fig 6. The most intense reaction was with constituents of the hair cortex while little reaction was noted with the root sheaths or medulla. Muscle was nonreactive.

Immunoprecipitation of Translation Products

The translation products encoded by the 18S fraction of RNA contained prominent species which were reactive with keratin antiserum. SDS-PAGE analysis of the immunoprecipitable products (Fig 7, *track 2*) demonstrated that proteins at 63K, 59K, 58K, 47K, and 46K *M*r were selectively precipitated from

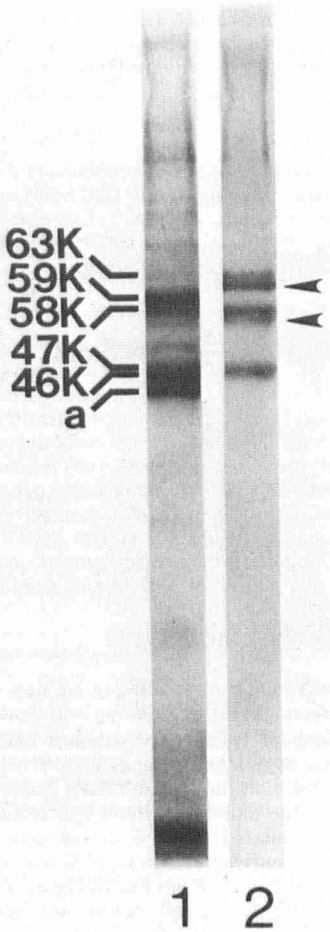

FIG 5. Reactivity of urea-extractable hair root proteins with keratin antiserum. ^{35}S-methionine-labelled, urea-extractable hair root proteins were fractionated into constituent components by SDS-PAGE. Proteins were then transferred to a nitrocellulose sheet by electrophoretic elution from the intact gel. Reactive bands were visualized by indirect immunostaining (see Methods). *1*, Autoradiogram of proteins transferred to nitrocellulose. *2*, Corresponding immunoelectrophoretogram. (*a*=actin).

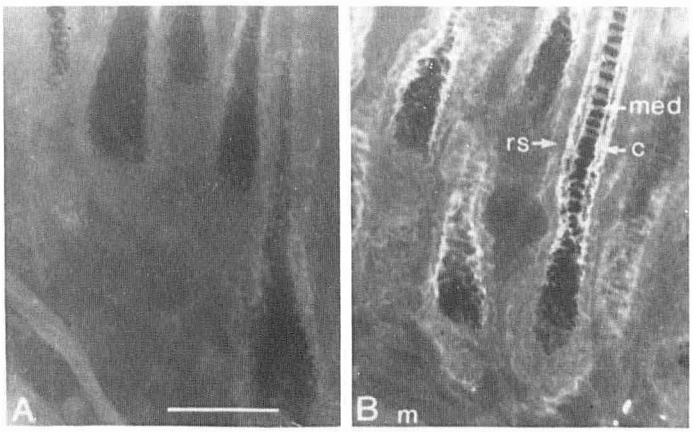

FIG 6. Distribution of antigens in the lower follicle reactive with keratin antiserum. Frozen sections (8 μ) of mouse skin were stained by the indirect immunofluorescence technique. *A*, Preimmune serum. *B*, Immune serum (*med*—hair medulla, *c*—hair cortex, *rs*—hair root sheaths, and *m*—muscle). *Scale bar*, 100 μ.

the pool of total translation products (*track 3*). No products were selectively precipitated by preimmune serum (*track 1*).

DISCUSSION

Indirect evidence [43] indicates that there are marked variations in the levels of individual hair root messenger RNAs during the hair cycle. It is our intention to make direct qualitative and quantitative measurements of individual messenger RNAs to determine their role in the control of mammalian hair growth. While this will require the use of recombinant DNA methodology, the first stage in such studies is the isolation of RNA at least partially enriched for the messenger RNAs of interest. Accordingly, we have developed a rapid and efficient means of isolating a fraction of skin enriched in anagen hair roots. Approximately 20-fold more RNA was obtained using this tissue fraction than was obtained from plucked hairs. *A priori*, the messenger pool in the RNA prepared from the hair root-enriched fraction might be expected to include a significant amount of contamination from nonfollicular sources such as muscle cells, adipocytes, and fibroblasts (Fig 1). However, translation products encoded by the 18S fraction of this RNA (enriched for species encoding proteins in the keratin molecular weight range) were qualitatively similar to those obtained with plucked hair root RNA (Fig 3, *tracks 4 and 3*, respectively). The differences in relative intensities of the bands in the two samples may be a consequence of a variety of factors including the extent of harvest of the deeper follicular material in addition to contamination from nonfollicular tissue.

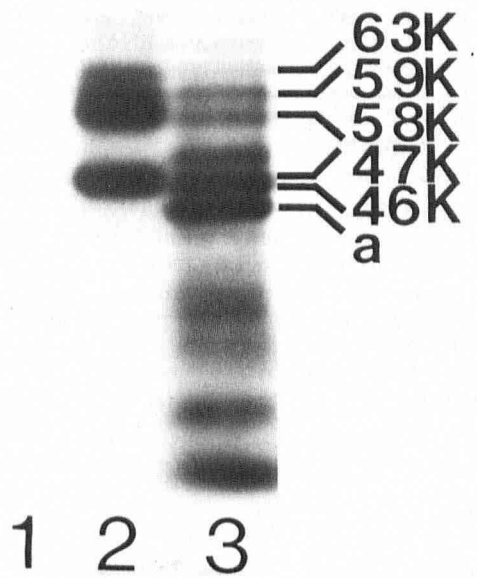

FIG 7. SDS-PAGE analysis of translation products immunoprecipitated with keratin antiserum. Bands were visualized by fluorography. *1*, Preimmune serum immunoprecipitate. *2*, Keratin antiserum immunoprecipitate. *3*, Total translation products encoded by 18S RNA. (*a*=actin).

Prominent translation products in the keratin molecular weight range with electrophoretic mobilities identical to authentic urea-extractable hair root proteins were noted at 63K, 59K, 58K, 47K, and 46K *M*r (Fig 3, *tracks 4 and 5*, respectively). Reactivity of the authentic proteins with keratin antiserum established that at least 4 of the 5 bands contained keratin. The 59K material did not react with the antiserum. It is unclear whether this 59K hair protein actually possessed distinct antigenic determinants which were nonreactive with the antiserum or if the antigenic determinants were particularly labile, such

that they were altered during the assay procedure. Since the keratin antiserum was prepared against epidermal keratins, reaction with the hair keratins was on the basis of cross-reactivity. Post-translational modification of the 59K hair protein *in vivo* might therefore be another potential explanation of the observed nonreactivity by the masking of antigenic determinants shared with the other keratins. Immunoprecipitation of translation products directly confirmed the presence of keratins (Fig 7) in all 5 bands. Thus, it is apparent that the 18S RNA isolated from the hair root-enriched skin fraction contains hair root keratin messenger RNAs.

Since adequate yields of RNA which encodes hair proteins have been obtained, this material is being purified further for use as a template for the synthesis of complementary DNA. "Probes" for specific messenger RNAs may then be produced by introduction of this material into a plasmid vector and propagation in a bacterial host, followed by selection of specific clones.

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